IDENTIFICATION OF AN ENDOCHITINASE cDNA CLONE FROM BARLEY ALEURONE CELLS

by

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INTRODUCTION

Effects of Gibberellic Acid

The effect of gibberellic acid 3 (GA) on barley aleurone tissue has long been used as a model system for the study of hormonal regulation of plant growth and development (Jacobsen, 1983). This tissue is amenable to laboratory study, and its response to GA includes changes in protein and RNA synthesis and the release of enzymes.

Himalaya barley seeds have as an outer layer a fused pericarp and testa which cover the aleurone. The aleurone is a layer three cells thick which surrounds the endosperm. Changes in aleurone cells after GA addition are directed toward making stored nutrients of the endosperm available for seedling growth (Palmer and Bathgate, 1976). GA induces increased activity, synthesis, or release of enzymes which degrade aleurone cell walls (Dashek and Chrispeels, 1977), endosperm cell walls (Mundy et al, 1985), nucleic acids (Brown and Ho, 1986), and storage carbohydrates (Chrispeels and Varner, 1967) and storage proteins (Hammerton and Ho, 1986) of the endosperm.

In one case GA induces an increase in the release of an enzymatic activity rather than new enzyme synthesis (Ashford and Jacobsen, 1974). Whether or not all new enzyme synthesis requires newly synthesized mRNA is not known. Some enzyme activities increase upon imbibition by half seeds. Some of these activities then increase even more after incubation with GA while others do not. The somewhat artificial imbibition and incubation procedures used in experiments to treat aleurones with and without GA make some results difficult to interpret when observed differences are small.

Table 1 shows the course of development of some enzymatic activities in barley aleurones and how GA affects this and the release of these activities.

Plant Chitinases

Chitinases are found in many higher plants and are generally present at low basal levels. Higher levels are observed in plants which are diseased, wounded, or treated with exogenous ethylene.

Cell walls of many fungi contain chitin as a major component (Bartnicki-Garcia, 1968). Plant chitinases are believed to be part of a defense mechanism against fungal attack (Boller, 1985). Reasons for this belief include the absence of chitin in higher plants and the induction of chitinase activity after infection with fungi or exposure to fungal cell wall components or sterilized spores. Bean chitinase also has a lysozyme activity, though its specific activity is only one-tenth that of egg white lysozyme when used on bacterial cell walls. Chitinase is induced in tobacco leaves infected with tobacco mosaic virus. This may be due to its being part of a plant response to pathogen attack since it has no obvious role in preventing viral infection.

While plant chitinases have not been shown to prevent or limit fungal attack in vivo, a number of studies indicate they possess anti-fungal properties. A crude extract from ethylene-treated bean leaves releases chitin oligosaccharides from isolated fungal cell walls (Boller et al, 1983). Bean leaf extracts and purified bean chitinase inhibit fungal growth on agar plates (Schlumbaum et al, 1986). An endochitinase

purified from wheat germ displays greater activity on newly formed chitin than on (synthetic) preformed chitin, possibly showing a greater activity on actively invading hyphae than on mature fungal cells (Molano et al, 1979).

Plant chitinases catalyze the hydrolysis of the beta-1,4 linkages of N-acetyl-D-glucosamine polymers (Broglie et al, 1986). The products of their activities range from free N-acetylglucosamine (NAG) to chitotetraose and higher oligosaccharides. A crude extract from bean leaves releases almost exclusively chitobiose, chitotriose, and chitotetraose within 30 minutes while free NAG and higher oligomers are also present. By 24 hours chitobiose and chitotriose comprise 87% of the species and higher oligomers 1% (Boller et al, 1983). A similar shift in products with time is observed with endochitinases from wheat germ (Molano et al, 1979) and melon seedlings (Roby and Esquerre-Tugaye, 1987). Detectable wheat germ endochitnase activity was absent or very minor with chitobiose and chitotriose, respectively, as substrates (Molano et al, 1979). Melon exochitinase releases only NAG and chitobiose (Roby and Esquerre-Tugaye, 1987).

Bean endochitinase has no detectable chitosanase, cellulase, beta-N-acetylglucosaminidase or beta-1,3-glucanase activities (Boller et al, 1983).

Hormonal Control of Chitinase

Exogenous ethylene treatment of dicot seedlings of seven different genera results in increased endochitinase activity ranging from 2.9 to 20-fold over control levels (Boller et al, 1983). Ethylene induction of plant chitinases has been studied in more detail in bean and melon

seedlings.

Bean seedlings treated with ethylene show a 30-fold increase in chitinase activity over control levels after 24 hours. Chitinase activity is absent in leaves treated with aminoethoxyvinylglycine (AVG), an inhibitor of ethylene synthesis in plants. The AVG effect is counteracted by addition of ethylene. Leaves incubated with 1-aminocyclopropane-1-carboxylic acid (ACC), the natural ethylene precursor, show a 4-fold increase in chitinase activity. Cycloheximide prevents ethylene induction of chitinase activity (Boller et al, 1983).

Treatment of bean seedlings with ethephon (2-chloroethylphosphonic acid, which upon non-enzymatic hydrolysis yields ethylene) causes a 75 to 100-fold increase in chitinase mRNA levels (Broglie et al, 1986).

Ethylene treatment of melon seedlings results in ethylene-induced increases in both exo- and endochitinase mRNA levels. Fungus-infected seedlings contain higher exo- and endochitinase mRNA levels than do healthy seedlings. Exo- and endochitinase activities are observed only in infected plants (Roby and Esquerre-Tugaye, 1987).

These results suggest that ethylene-induced chitinase activities are due to increased transcription of chitinase genes and subsequent translation of the mRNAs. Heat shock (40° C) and cold shock (0° C), which cause only transient bursts of ethylene production, do not induce chitinase activity (Boller, 1985).

Effects of cytokinin and auxin on tobacco chitinase have been examined, but contradictory results have been obtained. Cloned tobacco pith cells treated simultaneously with auxin and cytokinin contain lower levels of both chitinase activity and chitinase mRNA than control cells (Shinshi et al, 1987). Cells incubated with either hormone alone have

intermediate levels of chitinase activity. Chitinase content in normal plants is highest in root tissues and progressively declines in leaves from the bottom to the top of the plant, a distribution inversely correlated with auxin and cytokinin levels in plants.

By contrast, experiments with normal tobacco plants and plants transformed with a gene directing overproduction of cytokinin show higher levels of chitinase mRNA in the transformed plants (Memelink et al, 1987). The reason for this apparent discrepancy is not clear.

This study reports the identification of a cDNA clone for a barley aleurone endochitinase, the presence of chitinase activity in seed tissues and the effects of GA on the release of this activity.

MATERIALS AND METHODS

Barley DNA used for cloning was obtained from embryos dissected from Himalaya barley seeds (1985 harvest, Department of Agronomy, Washington State University). Clone 10 is a cDNA constructed from Himalaya barley aleurone mRNA and cloned into the Pst I site of pBR322 (Huang, 1986; Lee, 1987).

High molecular weight genomic DNA from barley embryos was isolated using the method of Blin and Stafford (1976) with the following changes. Tissue was homogenized with mortar and pestle at -70^{o} C. Extractions were done with chloroform and no RNase was used. After the first dialysis the extract was purified through two cycles of CsCl–ethidium bromide density gradient centrifugation. The sample then was extracted four times with CsCl–saturated butanol and extensively dialyzed against 10 mM Tris pH 8.0, 0.5 mM EDTA.

Restriction enzyme digestions were done for three or more hours under conditions recommended by the manufacturer (New England Biolabs) using up to a two-fold excess of enzyme.

The clone 10 fragments (Pst I and Pst I/Bal I) used for cloning into M13 were isolated by gel elution followed by spermine precipitation. Fragment concentrations were estimated by ethidium bromide stained gel analysis using spectrophotometrically measured amounts of linearized pBR322 DNA.

Vector DNA's (pUC 19, M13mp18, M13mp19) were digested with the appropriate restriction enzymes and then phenol and chloroform extracted and ethanol precipitated. Vectors were then treated with calf intestinal alkaline phosphatase (P-L Biochemicals) (Maniatis et al, 1982).

Ligation reactions were done at 12^{o} C using T4 DNA ligase (New England Biolabs) under the manufacturer's conditions in volumes of 5-10 ul. Total DNA concentrations were 5-10 ug/ml with insert:vector molar ratios of 3:1 to 10:1.

Competent cells were prepared using the JM 107 strain of E. coli and a protocol from the Pharmacia M13 cloning kit.

Single stranded DNA for sequencing was obtained from 100 ml cultures in 2X YT broth. After centrifugation at 6000 x g and 4^{o} C for 20 minutes, the supernatant was filtered through filter paper and then through a 0.45 um nitrocellulose membrane with vacuum suction. After addition of 25 ml of 25% PEG, 2.5M NaCl, the mixture was stored at 4^{o} C for 12 hours. This was centrifuged as before. The phage pellet was vacuum dried and suspended in 3 ml of 20 mM Tris pH 7.4, 0.5 mM EDTA, 10 mM NaCl. This was phenol/chloroform extracted and ethanol precipitated. After rinsing with 85% ethanol, the dried pellet was dissolved in 50 ul of 10 mM Tris pH8, 0.5 mM EDTA.

Sequencing was done using 35S-dATP (New England Nuclear, sp. act. 1300 Ci/mmole) and the dideoxy chain termination method (Sanger et al, 1977). The DNA polymerase ("Sequenase") and all reagents were used as recommended by the manufacturer (US Biochemical). Samples were electrophoresed at 60 watts, 1500 volts, 40 milliamps on 5% polyacrylamide gels containing 1X TBE, 2.5 mg/ml bis-acrylamide, 420 mg/ml urea.

Plant material prepared for chitinase assay consisted of embryoless half seeds surface-sterilized with 1% NaOCl and imbibed on sterile, moist sand at 4^{o} C for 3 days. Twenty half-seeds or dissected aleurone layers were incubated in 25 ml flasks containing 2 ml of 1 mM

NaOAc pH 4.8, 10 mM $CaCl_2$, 10 ug/ml chloramphenicol at 22° C and 125 cycles/minute in a metabolic shaker. The GA concentration was 1 uM.

Tissues were homogenized with a mortar and pestle using 0.2 g glass powder and 3 ml of 0.1 M KH_2PO_4 pH 6.5. The extract was centrifuged at 10° C and 9,000 x g for 10 minutes. These supernatants and the incubation media were kept at -20° C until used.

Tritiated chitin was prepared as described (Molano et al, 1977) except that after homogenization and washing, the chitin was baked at 80° C in a vacuum for 2 hours. The dry solid was ground with a mortar and pestle and suspended in 0.02% NaN_3 at 20 mg/ml. This was ground in a tissue homogenizer for 2 minutes and then washed seven times with 0.02 % NaN_3 after centrifugation for 2 minutes at 10,000 x g.

Each sample was assayed in triplicate in a volume of 100 ul in a solution containing 50 mM KH_2PO_4 pH 6.5, 0.5 mg tritiated chitin (80,000 cpm/mg), 0.75 mM NaN_3, and 0.25% of the sample (5.0 or 7.5 ul). After a 5 minute incubation at 37° C, the reactions were stopped by adding 300 ul of 10% TCA (w/v) and incubating on ice for 20 minutes. After the addition of 600 ul of 7.5% TCA, the sample was filtered through 0.8 um nitrocellulose. A 200 ul aliquot of the filtrate was added to 10 ml of scintillant (70 ml ethylene glycol, 280 ml ethanol, 1150 ml xylenes, 6 g PPO, 0.4 g POPOP, 500 ml Triton X-100) and counted in a Beckman Model LS 3801 liquid scintillation counter. Samples were counted three times for 10 minutes with a window setting of 0-400.

The specific activity of the substrate was determined after complete digestion with HCl (Rupley, 1964).

RESULTS

Sequence of Clone 10 and Its Protein Product

The nucleotide sequence of the clone 10 insert contains 556 bp of barley sequences. One strand contains the only long open reading frame. This ORF begins at the 5' end of the clone and stops 22 nucleotides from the 3' end. Stop codons for the other two possible reading frames closely follow that of the ORF. The consensus polyadenylation signal sequence is not present.

The nucleotide sequences from both strands of clone 10 were used to search for similar sequences in the data banks maintained by or accessed through the National Biomedical Research Foundation. The search was done with the ALIGN program (Lipman and Pearson). The strand containing the long ORF showed substantial similarity to the 3' ends of the coding strands of endochitinase cDNAs from *Phaseolus vulgaris* (Broglie et al, 1986) and *Nicotiana tabacum* (Shinshi et al, 1987) (Figure 1). The identity of the *Phaseolus* endochitinase cDNA clone had been shown by immunoprecipitation of the translation products of mRNA hybrid-selected by the cDNA.

Translation of the clone 10 ORF predicts a sequence of 178 amino acids. This sequence and those deduced from the ORFs of the bean and tobacco cDNAs plus that of a cyanogen bromide fragment of a barley endochitinase are shown in Figure 2.

The similarity of the clone 10 nucleotide sequence with those reported for endochitinase cDNA's from bean and tobacco is summarized in Table 2. These comparisons include only the regions corresponding to clone 10's open reading frame. The clone 10 DNA sequence is identical

to both the bean and tobacco sequences in about 65% of the positions. About 60% of the mismatches in each comparison are at the third position of individual codons (Table 3).

Alignment of the three deduced amino acid sequences is shown in Figure 2. The barley sequence has residues identical to those of bean and tobacco at 74% of the positions (Table 4). The nucleotide and predicted amino acid sequences of the bean and tobacco cDNA clones share 67% and 73% identities, respectively (Shinshi et al, 1987). The plant amino acid sequences exhibit no similarity to that of a bacterial endochitinase from *Serratia marcescens* (Jones et al, 1986).

Chitinase Activity in Barley Seeds

Tritiated chitin was used as a substrate to detect chitinase activity in tissue extracts and incubation media of embryoless half seeds and isolated aleurone layers. A time course assay showed a profile consistent with that of an enzymatic activity (Figure 3). Activities only 10% or less than maximal were observed when the sample was boiled for five minutes prior to the assay. These assays measured the total chitinase activity present and may include activities other than that encoded by clone 10.

Assays of tissue extracts from half seeds which had only been surface-sterilized showed chitinase activity in both the aleurone and endosperm but not in the pericarp. Comparable activities were present after imbibition (Table 5).

Assays of tissue extracts after 24 and 48 hour incubations showed a

progressive loss of activity from the tissues with time (Table 5). This loss was greater in the presence of GA for both half seeds and isolated aleurones.

Assay of aliquots of media at 12 hour intervals showed a progressive increase with time in chitinase activity in the control samples but little change between 24 and 48 hours in GA-treated samples (Table 5). With one exception, the activities were always greater in GA-treated samples.

Results on the effects of different plant hormones on the release of chitinase activity into the medium are shown in Table 5. Neither ethylene (gas) nor kinetin (a synthetic cytokinin) stimulated more release than that measured from half seeds incubated without hormone.

Compared to the zero time values for endosperm and aleurone prior to incubation, the total activities detected for the tissues plus the media were greater at the end of both 24 and 48 hour incubations, possibly indicating net synthesis during the incubation.

DISCUSSION

I propose that clone 10 represents a barley endochitinase gene. This is based on the similarities of its nucleotide and deduced (ORF) amino acid sequences with those reported for bean and tobacco endochitinase cDNAs. The even stronger similarity with the amino acid sequence of the barley endochitinase (protein C) fragment further suppports this identification. Two related features of clone 10 are like those of the bean cDNA which hybridizes to a 1.2 kb mRNA that produces a 35 kD primary translation product (Broglie et al, 1986). The respective values for clone 10 are 1.4 kb and 36 kD (Lee, 1987).

The non-identity of the clone 10 and protein C amino acid sequences might mean that there are two different barley endochitinases. This difference could be more apparent than real if due to differences in nucleotide versus amino acid sequencing or if because they are derived from seeds of different barley cultivars and are simply polymorphic enzymes derived from the same locus. Isolation and sequencing of several endochitinase cDNA clones could resolve this issue.

If both proteins are present in each variety, it could reflect the importance of having endochitinases with activities that differ in some way. Protein C is synthesized by the endosperm during seed maturation (Mundy et al, 1986). The clone 10 gene is expressed in aleurone cells (Huang, 1986; Lee, 1987).

The first study of a barley endochitinase (Leah et al, 1987) reported that the protein was from the endosperm. Prior to the identification of protein C as an endochitinase, mRNA for this protein was detected in the endosperm but not in the aleurone of developing

seeds or in aleurones incubated with and without GA (Mundy et al, 1986). In that study, antibodies to protein C were used to precipitate in vitro translation products of the mRNAs isolated from these tissues. Unless antibodies to protein C do not recognize the protein encoded by clone 10, their result differs from those obtained in the present study.

The chitinase assays described here are the first report of the presence of chitinase activity in barley aleurone cells and in the media from seed incubations. The activity in the aleurone layers detected in these experiments cannot only be due to contamination from what little endosperm remained associated with the dissected aleurone layers. The levels of activity in the aleurone were greater than those in the endosperm in every experiment, even though in half seeds the dry weight of the endosperm is 2.4 times greater than that of the aleurone. As a percentage of the dry weight, chitinase activity is greater in the aleurone than in the endosperm.

Chitinase activity was found in both the aleurone and endosperm of mature seeds. The activities were unchanged in these tissues after imbibition for three days. This suggests that there is no new synthesis of chitinase during imbibition.

The presence of chitinase activity in mature seeds might protect dormant seeds from fungal attack. Chitinases may also have anti-bacterial properties (Boller, 1985). The effects of plant chitinases on insects have not been studied.

Upon incubation of half-seeds or isolated aleurones, activity in the tissues decreased and was released to the medium. It is not known if this is the result of an active secretion process or a passive loss from the aleurone layer cells as a result of the enzymatic degradation

of aleurone cell walls during germination. The activity loss from the endosperm must be a passive process because these cells are dead in mature seeds. Compared to control samples, the greater loss from tissues treated with GA must be at least partly the result of the GA-stimulated release of enzymes that degrade aleurone and endosperm cell walls.

The assays show that chitinase released to the medium retains at least some of its activity. Chitinases also show anti-fungal activity outside of cells. A bean endochitinase inhibits fungal growth on agar plates (Schlumbaum et al, 1986) as does a barley protein thought to be protein C (Roberts and Selitrennikoff, 1986; Leah et al, 1987). The release of chitinase activity from barley seeds in the simulated germinations described here might indicate an important activity that protects sprouting seeds and seedlings from fungal attack.

Chitinase activity in the control medium increased with time through 48 hours. The time course sampling of incubation media from control half seeds and aleurones showed greater activity loss from the isolated aleurones than from the half seed aleurone and endosperm combined, even though the two aleurone tissue samples retained almost equal amounts of activity. It is possible that the total activity present at 48 hours represents some new synthesis of chitinase enzymes by the aleurones and that this synthesis began earlier in the isolated aleurones. This could be the result of the dissection to prepare isolated aleurones for incubation. A large and rapid increase in chitinase mRNA following excision wounding of bean hypocotyls has been reported (Hedrick et al, 1988).

The time course sampling of incubation media from GA-treated half

seeds and aleurones showed a levelling off of chitinase activity after 24 hours. At 12 hours more had been released from isolated aleurones than half seeds, possibly because of wound induction. Activities declined slightly for half seed samples between 36 and 48 hours and for isolated aleurones between 24 and 48 hours. This could be due to insensitivity of the assays or could be the result of the GA-dependent continual increase in the release of proteases and carboxypeptidase between 24 and 48 hours (Hammerton and Ho, 1986). The proteases can digest hordein, gliadin, and hemoglobin.

At all time points except one, more chitinase activity was detected in the medium of GA-treated samples than in the controls. GA appears to hasten the onset of chitinase activity release and to have no detectable effect on the total amount of chitinase activity present.

Assays of media from control, GA-, ethylene-, and kinetin-treated half-seeds showed that only GA stimulated the release of chitinase activity. Ethylene induced chitinase activity in young plants of several different genera but not in wheat (Boller et al, 1983).

The total amount of chitinase activity present in the tissues and media after 24 and 48 hour incubations was always greater than that present in the tissues prior to incubation. This suggests aleurones may synthesize proteins with chitinase activity during incubation.

The existence of active endochitinase enzymes in mature barley seeds and the apparent increase in chitinase activity under aseptic germination conditions indicate the importance of protecting dormant and germinating seeds from fungal infection. Seeds contain limited materials available for seedling growth, and allocation of these resources must be closely controlled.

Figure 1a: Nucleotide Sequence Alignment

Upper sequence is clone 10. Lower sequence is Phaseolus endochitinase cDNA.

```
          X        10        20        30        40
          CACGAGACCACCGGCGGGTGGGCGACGGCACCGGACGGAGCTTTCGCCTG
          ::::: :: ::::: :: ::::: :: :: :: :::::: : : ::: ::
GCAAACGTCTCACGAAACAACCGGGGGATGGGCCACTGCGCCCGACGGACCATACGCATG
X        10        20        30        40        50

         60        70        80        90        100
GGGCTACTGCTTCAAGCAGGAGCGTGGCGCCACCTCCAACTACTGCACTCCGAGCGCGCA
::: :::::::::  :  ::::::   : ::: : :    :::::: :  : :   : ::
GGGATACTGCTTCGTGAGGGAGCGGAACCCCAGCACG---TACTGCTCCGCCACTCCCCA
         70        80        90           100       110

         120       130       140       150       160
GTGGCCGTGCGCCCCAGGGAAGAGCTACTACGGCCGTGGGCCGATCCAGCTCTCCCACAA
::  :: :::::::: :::: ::   ::::::::: : :: :: :::::: : :::   ::
GTTCCCCTGCGCCCCTGGGCAGCAGTACTACGGCAGGGGTCCCATCCAGATATCCTGGAA
  120       130       140       150       160       170

         180       190       200       210       220
CTACAACTACGGGCCTGCGGGCCGGGCTATAGGGGTCGATCTGCTGCGCAACCCGGACCT
:::::::::::: :     ::  :::: :: ::::: ::  ::::   ::: :: :: ::
CTACAACTACGGTCAGTGCGGAAGGGCCATTGGGGTTGACTTGCTCAACAAACCTGATCT
  180       190       200       210       220       230

         240       250       260       270       280
GGTGGCCACGGACCCGACCGTGTCGTTTAAGACTGCGATGTGGTTTTGGATGACGGCCCA
 :: ::::: ::: :   : : :: :: ::: : ::  : ::::: :::::::: :: ::
AGTCGCCACTGACTCTGTCATCTCCTTCAAGTCCGCCCTCTGGTTCTGGATGACCGCACA
  240       250       260       270       280       290

         300       310       320       330       340
GGCGCCAAAACCGTCGAGCCATGCTGTGATCACGGGCCAGTGGAGCCCATCAGGGACGGA
: : :: :: :: ::   ::: :  :: :::::    :  :::: ::: ::     : ::
GTCCCCCAAGCCTTCCTCCCACGACGTCATCACCTCTCGATGGACCCCCTCCTCTGCCGA
  300       310       320       330       340       350

         360       370       380       390       400
CCGGGCCGCGGGGCGGGTGCCTGGGTTTGGCGTGATCACCAACATCGTCAACGGCGGGAT
:   :::::  : ::: : :: :: :  :::    : :: :::::: ::::::: ::  :
CGTCGCCGCCCGCCGGCTTCCCGGCTACGGCACTGTGACGAACATCATCAACGGAGGCCT
  360       370       380       390       400       410

         420       430       440       450       460
CGAGTGCGGGCATGGGCAGGACAGTCGAGTCGCCGATCGGATCGGGTTTTACAAGCGCTA
 ::::::::::  :: ::::::::  : ::    :: :: ::::: :: : :::: : ::
GGAGTGCGGGCGAGGACAGGACAGCAGGGTTCAAGACCGCATCGGATTCTTCAAGAGATA
  420       430       440       450       460       470

         480       490       500       510       520
CTGCGACATCCTCGGCGTTGGCTACGGCAACAACCTCGACTGCTACAGCCAGAGGCCCTT
::: ::  : :: :: :: :: :: ::::::::::: :::::::::   ::::  :: ::
CTGTGATCTGCTTGGAGTCGGTTATGGCAACAACCTTGACTGCTACTCTCAGACTCCATT
  480       490       500       510       520       530
```

Figure 1b: Nucleotide Sequence Alignment

Upper sequence is clone 10. Lower sequence is Nicotiana endochitinase cDNA.

```
  X        10        20        30        40        50
  CACGAGACCACCGGCGGGTGGGCGACGGCACCGGACGGAGCTTTCGCCTGGGGCTACT
  :: :: :: :: :: :: ::::: :: ::::: :: ::  : : ::: ::::: ::::
CCCATGAAACTACAGGAGGATGGGCAACAGCACCAGATGGTCCATACGCGTGGGGTTACT
X        10        20        30        40        50

 60        70        80        90        100       110
GCTTCAAGCAGGAGCGTGGCGCCACCTCCAACTACTGCACTCCGAGCGCGCAGTGGCCGT
:::        :: :  ::   : ::  : ::::::: :: :: :: :  :::::::: :
GCTGGCTTAGAGAACAAGGTAGCCCCGGCGACTACTGTACACCAAGTGGTCAGTGGCCTT
         70        80        90        100       110

 120       130       140       150       160       170
GCGCCCCAGGGAAGAGCTACTACGGCCGTGGGCCGATCCAGCTCTCCCACAACTACAACT
: :: :: ::   ::  :: : ::: :: :: :: :::::  : :: :::::::::::::
GTGCTCCTGGTCGGAAATATTTCGGACGAGGCCCCATCCAAATTTCACACAACTACAACT
         130       140       150       160       170

 180       190       200       210       220       230
ACGGGCCTGCGGGCCGGGCTATAGGGGTCGATCTGCTGCGCAACCCGGACCTGGTGGCCA
:::: :::   ::  : :: ::::: :: :: :: ::   ::: :: ::  : :::::::
ACGGACCTTGTGGAAGAGCCATAGGAGTGGACCTCCTAAACAATCCTGATTTAGTGGCCA
         190       200       210       220       230

 240       250       260       270       280       290
CGGACCCGACCGTGTCGTTTAAGACTGCGATGTGGTTTTGGATGACGGCCCAGGCGCCAA
: :: ::     : :: :: ::: : ::  : ::::::::::::::  : ::  : ::::
CAGATCCAGTAATCTCATTCAAGTCAGCTCTCTGGTTTTGGATGACTCCTCAATCACCAA
         250       260       270       280       290

 300       310       320       330       340       350
AACCGTCGAGCCATGCTGTGATCACGGGCCAGTGGAGCCCATCAGGGACGGACCGGGCCG
:::: ::  :::: : ::: ::::  ::    :::   :::::     : ::::: :: :
AACCTTCTTGCCACGATGTCATCATTGGAAGATGGCAACCATCGTCTGCTGACCGCGCAG
         310       320       330       340       350

 360       370       380       390       400       410
CGGGGCGGGTGCCTGGGTTTGGCGTGATCACCAACATCGTCAACGGCGGGATCGAGTGCG
:    ::  : ::::: ::::: :: ::::: :::::: :::: :: ::  : :: :: :
CCAATCGTCTCCCTGGATTTGGTGTCATCACGAACATCATCAATGGTGGCTTGGAATGTG
         370       380       390       400       410

 420       430       440       450       460       470
GGCATGGGCAGGACAGTCGAGTCGCCGATCGGATCGGGTTTTACAAGCGCTACTGCGACA
: : :::    :::    : :::   ::::: :: :::::::::: : : :: :::   :
GTCGTGGCACTGACTCAAGGGTCCAGGATCGCATTGGGTTTTACAGGAGGTATTGCAGTA
         430       440       450       460       470

 480       490       500       510       520       530
TCCTCGGCGTTGGCTACGGCAACAACCTCGACTGCTACAGCCAGAGGCCCTTCGCTTAAC
: :: :: ::: :    ::  :::: :: :: :::   : :::::::  :::     :::
TTCTTGGTGTTAGTCCTGGTGACAATCTTGATTGCGGAAACCAGAGGT-CTTTTGGAAAC
         490       500       510       520       530
```

Figure 2: Amino Acid Sequence Alignment

First line is clone 10 nucleotide sequence. Barley sequence is that of protein C (endochitinase) cyanogen bromide fragment. Others are deduced from cDNA nucleotide sequences. "*" indicates residues identical to those predicted for clone 10. ">" indicates stop codon.

```
CACGAGACCACCGGCGGGTGGGCGACGGCACCGGACGGAGCTTTCGCCTGGGGCTACTGC   60
H  E  T  T  G  G  W  A  T  A  P  D  G  A  F  A  W  G  Y  C      20
Clone 10
*  *  *  *  *  *  *  *  *  *  *  *  *  P  Y  *  *  *  *  *
Bean
*  *  *  *  *  *  *  *  *  *  *  *  *  P  Y  *  *  *  *  *
Tobacco

TTCAAGCAGGAGCGTGGCGCCACCTCCAACTACTGCACTCCGAGCGCGCAGTGGCCGTGC  120
F  K  Q  E  R  G  A  T  S  N  Y  C  T  P  S  A  Q  W  P  C      40
Clone 10
*  V  R  *  *  N  P  -  *  T  *  *  S  A  T  P  *  F  *  *
Bean
W  L  R  *  Q  *  S  P  G  D  *  *  *  *  *  G  *  *  *  *
Tobacco

GCCCCAGGGAAGAGCTACTACGGCCGTGGGCCGATCCAGCTCTCCCACAACTACAACTAC  180
A  P  G  K  S  Y  Y  G  R  G  P  I  Q  L  S  H  N  Y  N  Y      60
Clone 10
*  *  *  Q  Q  *  *  *  *  *  *  *  *  I  *  W  *  *  *  *
Bean
*  *  *  R  K  *  F  *  *  *  *  *  *  I  *  *  *  *  *  *
Tobacco

GGGCCTGCGGGCCGGGCTATAGGGGTCGATCTGCTGCGCAACCCGGACCTGGTGGCCACG  240
G  P  A  G  R  A  I  G  V  D  L  L  R  N  P  D  L  V  A  T      80
Clone 10
*  Q  C  *  *  *  *  *  *  *  *  *  N  K  *  *  *  *  *  *
Bean
*  *  C  *  *  *  *  *  *  *  *  *  N  *  *  *  *  *  *  *
Tobacco

GACCCGACCGTGTCGTTTAAGACTGCGATGTGGTTTTGGATGACGGCCCAGGCGCCAAAA  300
D  P  T  V  S  F  K  T  A  M  W  F  W  M  T  A  Q  A  P  K     100
Clone 10
*  S  V  I  *  *  *  S  *  L  *  *  *  *  *  *  *  S  *  *
Bean
*  *  V  I  *  *  *  S  *  L  *  *  *  *  *  P  *  S  *  *
Tobacco
                                         *  *  *  *  P  *  *
Barley
CCGTCGAGCCATGCTGTGATCACGGGCCAGTGGAGCCCATCAGGGACGGACCGGGCCGCG  360
P  S  S  H  A  V  I  T  G  Q  W  S  P  S  G  T  D  R  A  A     120
Clone 10
*  *  *  *  D  *  *  *  S  R  *  T  *  *  S  A  *  V  *  *
Bean
*  *  C  *  D  *  *  I  *  R  *  Q  *  *  S  A  *  *  *  *
Tobacco
*  *  *  *  *  *  *  A  *  *  *  *  *  D  *  A  *  *  *  *
Barley

GGGCGGGTGCCTGGGTTTGGCGTGATCACCAACATCGTCAACGGCGGGATCGAGTGCGGG  420
G  R  V  P  G  F  G  V  I  T  N  I  V  N  G  G  I  E  C  G     140
Clone 10
```

Figure 3: Time Course Chitinase Assay

Assay of medium from 24 hour incubation of GA-treated half seeds. Aliquots removed at times indicated. Value at 720 minutes (not shown) is 504 nkat. One nkat = nmoles NAG equivalents released per second.

Figure 3

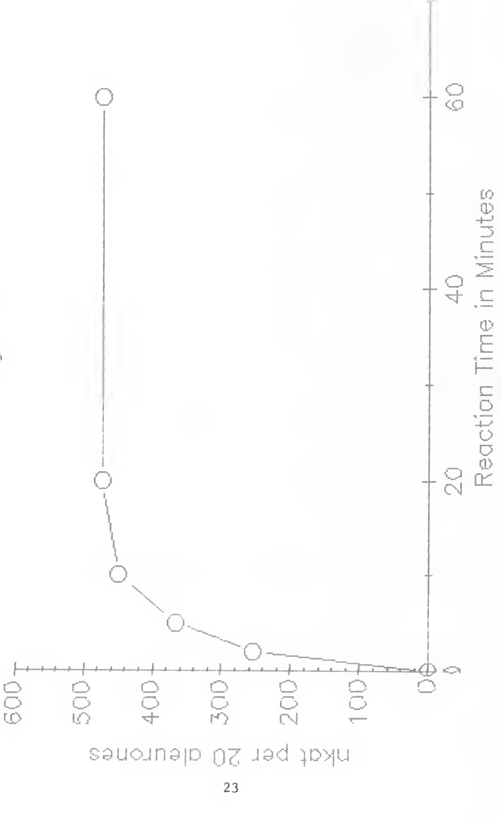
nkat per 20 aleurones
600
500
400
300
200
100
0
0
20
40
60
Reaction Time in Minutes

Table 1: Gibberellic Acid Effects on Barley Aleurone Enzyme Activities

Y indicates yes, N indicates no, and – indicates not determined.

Table 1 Gibberellic Acid Effects on Barley Aleurone Enzyme Activities

Enzyme	Activity in Mature Seed	Increase During Inbibition	Increase During Incubation Greater		Release into Medium Greater		New Enzyme Synthesis Greater		New Transcription Greater		Reference
			No GA	with GA	No GA	with GA	No GA	with GA	No GA	with GA	
Acid Phosphatase	Y	Y	Y	N	Y	Y	Y	N	–	–	1,2
Nuclease	–	–	Y	Y	N	Y	Y	Y	–	–	9,10
Endo-B-1,4-Xylanase	–	–	N	Y	N	Y	–	–	–	–	40
Xylopyranosidase	–	–	N	Y	N	Y	–	–	–	–	40
a-Arabinofuranosidase	–	–	N	Y	N	Y	–	–	–	–	40
B-1,3-Glucanase	–	Y	Y	N	N	Y	Y	–	–	–	4,17
1,3-1,4-B-D-glucanase	–	–	Y	Y	Y	Y	–	–	–	–	39
Carboxypeptidase	N	Y	Y	Y	N	Y	–	–	–	–	13,24
Endopeptidase	–	–	N	Y	N	Y	–	–	–	–	13,24
a-Amylase	–	–	Y	Y	N	Y	Y	Y	Y	Y	27,30

Table 2: Summary of Nucleotide Sequence Alignments

Number of positions at which bean and tobacco sequences are identical to that of clone 10.

Table 2

Nucleotide Sequence Alignment

Identities in Clone 10 Sequence vs:

Bean	364/556	65%
Tobacco	357/556	64%

Table 3: Nucleotide Sequence Mismatches by Codon Position

Comparisons include only the sequences within the clone 10 open reading frame.

Table 3

Nucleotide Sequence Mismatches by Codon Position

Clone 10 Sequence vs:

Codon Position	Bean	Tobacco
1	41 (23%)	39 (21%)
2	29 (17%)	30 (16%)
3	105 (60%)	118 (63%)

Table 4: Amino Acid Sequence Alignments

Comparisons include only the sequences within the clone 10 open reading frame.

Table 4

Amino Acid Sequence Alignments

Identities in Clone 10 Sequence vs:

Bean	131/178	74%
Tobacco	132/178	74%
Protein C	36/41	88%

Table 5: Chitinase Assays

Values reported are nkat per 20 half seeds. One nkat equals nmoles NAG equivalents released per second. GA and kinetin concentrations equal 1 uM. Ethylene concentration equals 10 nl per ml.

Table 5 Chitinase Assays

	Dry Half Seeds	Imbibed Half Seeds	24 Hour Half Seed Medium: Control	GA	Ethylene	Kinetin
Aleurone	300	310	130	320	130	110
Endosperm	240	250				
Total	540	560				

	24 Hour Incubation: Half Seeds C	Half Seeds GA	Isolated Aleurones C	Isolated Aleurones GA	48 Hour Incubation: Half Seeds C	Half Seeds GA	Isolated Aleurones C	Isolated Aleurones GA
Aleurone	280	200	300	290	240	140	240	180
Endosperm	220	190	—	—	200	80	—	—
Final Medium	220	320	110	260	210	360	310	300
Total	720	710	410	550	650	580	550	480

12 Hour Medium	10	130	140	280
24 Hour Medium	90	360	250	340
36 Hour Medium	190	380	290	310
48 Hour Medium	210	360	310	300

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IDENTIFICATION OF AN ENDOCHITINASE
cDNA CLONE FROM BARLEY ALEURONE CELLS

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AN ABSTRACT OF A THESIS

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ABSTRACT

A barley cDNA clone (# 10) isolated from a library constructed from RNA from gibberellic acid-treated aleurone cells was initially identified as a gibberellic acid-inducible clone. The nucleotide sequence of this clone was determined. A computer search of a nucleic acid sequence database revealed that this cDNA is closely related to endochitinase cDNA clones from bean and tobacco. Chitinase activity was detected in extracts of aleurone layers and the endosperm and in the incubation medium of half seeds incubated in the presence and absence of gibberellic acid. Enzyme assays showed that gibberellic acid caused a decrease in chitinase activity in seed tissues and promoted the release of this activity to the incubation medium.